DEVELOPMENTAL BIOLOGY 178, 35–50 (1996)
ARTICLE NO. 0196

That Cooperates with Grk TGFα in the Genesis of the Follicular Epithelium

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brainiac (brn) is involved in a number of developmental events. In addition to being required zygotically for segregation of neuroblasts from epidermoblasts, it is essential for a series of critical steps during oogenesis which also depend upon *gurken (grk),* a TGFα homolog. Animals harboring strong mutations of either *grk* or EGF receptor tyrosine kinase (Egfr) or doubly mutant for *brn* and weak *grk* or *Egfr* mutations produce ovarian follicles with multiple sets of nurse cell–oocyte complexes. These follicles frequently have discontinuities in the follicular epithelium that uncover nurse cells but not the oocyte. Gaps first appear in the germarium, suggesting that some nurse cells lack affinity for invading prefollicular cells. This is the first evidence that *grk,* in addition to its involvement in the genesis of anterior–posterior and dorsal–ventral polarity, is also required for Egfr-dependent development of the follicular epithelium that surrounds each nurse cell/oocyte cluster to form an egg chamber. We have used restriction fragment length polymorphisms to localize *brn* to a 10-kb region within a 300-kb stretch of DNA on the X-chromosome, and we have identified the *brn* gene by means of RNA rescue. *brn* codes for a putative secreted protein. *brn* is expressed in germ cells at the time follicle cells first surround the nurse cell–oocyte complex. Our genetic data suggest that *brn* acts in a parallel, but partially overlapping pathway to the Grk–Egfr signaling pathway. The *brn* pathway may help to provide specificity to TGFα–Egfr function during oogenesis.

INTRODUCTION

The Notch family of receptors regulates several types of cellular interactions in a wide variety of multicellular organisms (reviewed in Artavanis-Tsakonis *et al.,* 1995). Insight into Notch function in *Drosophila melanogaster* has been facilitated by the characterization of additional loci whose loss of function produces similar phenotypes to a loss of Notch function. These genes have been termed ''neurogenic'' because in their absence, the embryonic ectoderm develops supernumerary neuroblasts at the expense of epidermoblasts (Lehmann *et al.,* 1983; reviewed in Campos-Ortega and Knust, 1990). In embryonic ectoderm as well as other tissues, the neurogenic genes have been shown to act via lateral specification to regulate bipotential cell fate decisions (Cajan and Ready, 1989; Hartenstein and Posakony, 1989; Corbin *et al.,* 1991; Ruohola *et al.,* 1991).

Another type of cellular interaction regulated by neurogenic gene function involves the development of several embryonic epithelia in which no obvious decision between alternative cell fates is being made (Hartenstein *et al.,* 1992). We are exploring the role of neurogenic genes in epithelial development by studying their function in establishing and maintaining the epithelium that surrounds the ovarian follicle (see Spradling, 1993, for a review of *Drosophila* oogenesis). We have previously shown that the neurogenic gene *brainiac* (*brn*) has a novel germline activity essential for the development of the follicular epithelium (Goode *et al.,* 1992). *brn* is unusual in other respects. *brn* differs from the primary neurogenic genes in that it does not affect bristle number or other aspects of the differentiation of the pupal ectoderm that require lateral specification. Further, in contrast to other neurogenic genes, *brn* mutations do not show dosage sensitive interactions with Notch mutations during oogenesis or neurogenesis (Goode, 1994). In contrast, we have shown that germline *brn* cooperates with the *Drosophila* EGF receptor tyrosine kinase (Egfr), expressed in the follicular epithelium (Price *et al.,* 1989; Schejter and Shilo,

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0012-1606/96 $18.00

1989), to regulate at least three follicle cell activities essential for the morphogenesis of the follicular epithelium: the migration of the prefollicular cells around each ovarian follicle, the establishment of a complete follicular epithelium, and the development of dorsal follicular cell fates (Goode *et al.*, 1992).

The role of the Egfr in the establishment of the dorsal–ventral (Schüpbach, 1987) and anterior–posterior (González-Reyes *et al.*, 1995; Roth *et al.*, 1995) polarity of the egg shell and embryo during oogenesis has been described in detail (reviewed in Lehmann, 1995). A putative ligand for Egfr is the product of the *gurken* (*grk*) locus, a TGFα-like molecule (Neumann-Silberberg and Schüpbach, 1993). During the early stages of oogenesis, *grk* RNA and protein are localized to the posterior of the oocyte and are required for induction of posterior polar cell fate (Roth *et al.*, 1995). Polar cell determination is essential for the establishment of anterior–posterior polarity within the oocyte (Ruohola *et al.*, 1991). At a subsequent step, when vitellogenesis begins, the oocyte nucleus becomes localized to the anterior–dorsal border of the oocyte and *grk* mRNA and protein become concentrated between the nucleus and the oolemma (Neuman-Silberberg and Schüpbach, 1993). The temporal and spatially distinct localizations of *grk* ensure activation of the Egfr in specific subsets of follicle cells. Thus, the Egfr plays a critical role in the specification of posterior polar cells and anterior dorsal follicle cells, respectively.

In this study, we show that *grk* plays a third role in the development of the follicular epithelium by cooperating with *brn* for the Egfr-dependent migration of the prefollicular cells around each nurse cell–oocyte complex. We have cloned *brn* and shown that it codes for a putative secreted protein. *brn* is first expressed in germ cells at the time that follicle cells begin to surround the germ cells. Simultaneous reduction of *brn* and *grk* function during oogenesis leads to failure to segregate each nurse cell–oocyte cluster within its own follicular epithelium and to dramatic discontinuities in this epithelium. Based on our genetic analysis of *brn* cooperativity with *grk*, we propose that *brn* may help provide specificity to and/or facilitate the multiplicity of Grk–Egfr functions during oogenesis.

MATERIALS AND METHODS

Genetics. All strains were grown at 25°C on standard *Drosophila* medium. All *Egfr* mutations are described in Clifford and Schüpbach (1994). *grk* mutations are described in Neumann-Silberberg and Schüpbach (1993). *brn* mutations are described in Goode *et al.* (1992).

Df(1)cho^{L1} was synthesized by irradiating *white* males with 4000–5000 rad (γ rays) and crossing to *yellow(y) chocolate*(*cho*) virgins. Female progeny with chocolate-colored eyes were mated to *FM7a* males. Complementation was tested by standard methods.

Gaps in follicular epithelium. Ovaries were stained with phalloidin by fixing in 4% formaldehyde in PBS, followed by teasing apart ovarioles before addition of fluorescein phalloidin (Molecular Probes) at a concentration of 0.16 μg/ml in PBS (1 hr).

For electron microscopy, egg chambers were fixed in 2% glutaraldehyde (prepared in PBS, pH 7.4) as described in Mahowald *et al.* (1979).

Nucleic acids. Miniprep genomic, plasmid, and cosmid DNA was prepared as described in Bender *et al.* (1983) or Sambrook *et al.* (1989). Ultrapure CsCl genomic DNA and cDNA (for *in vitro* transcription) was prepared as described by R. Lifton (personal communication). λ phage DNA was prepared essentially as in Davis *et al.* (1983). Since cDNAs had been directionally cloned (Brown and Kafotos, 1988), restriction analysis was sufficient to establish the transcriptional orientation of the corresponding transcripts.

mRNA for Northern analysis was prepared with a RiboSep mRNA isolation kit (Becton–Dickinson), from approximately 5 g of fly embryos per developmental stage. mRNA for injections was synthesized from linearized cDNA vectors as described in Krieg and Melton (1987), and capped with G(5′)ppp(5′)G (New England Biolabs). mRNA was selectively precipitated with ethanol. The size and stability of the mRNA was confirmed by gel electrophoresis.

Filters and hybridizations. Southern blots and plaque lifts and replica filters for genomic, cDNA, and other experiments were prepared according to standard protocols (Sambrook *et al.*, 1989; J. W. Tamkun, personal communication). To map deficiency breakpoints, approximately equal quantities of male and female control DNAs and female experimental DNAs were loaded for each genotype (judged by ethidium bromide fluorescence). Probe DNA was determined to reside within the deficiency if signal equaled male control signal, outside the deficiency if the signal equaled female control signal, and to span a deficiency breakpoint if novel restriction fragments were detected. Control probes of known dosage were used to confirm uncertain results.

For Northern blots, mRNA was electrophoresed (1% gels) and blotted essentially as described in Sambrook *et al.* (1989). UV-crosslinked (12 mJ) filters were probed (^{32}P-labeled DNA) in Denhardt's-based solutions and washed at $T_m = -10$°C. YAC DNA was digested with *Eco*RI before labeling.

Biotinylated probe (BRL BioNick nick-translation system) was used for *in situ* hybridizations to chromosomes. Cosmid 57 had been reported to hybridize to region 3F by the Crete Genome Project. For *in situ* hybridizations to ovarian tissue, the protocol of González-Reyes and St. Johnston (1994) was followed.

λ*rbJN isolation.* A *Df(1)rb*^{46} genomic library was constructed (λ EMBL4 arms, Gigapack II Packaging Extract (Stratagene)), plated, and screened using developed methodologies (Sambrook *et al.*, 1989). The library was screened with a 3.7-kb *Bam*HI fragment of λIX.27, immediately proximal of the *Df(1)rb*^{46} breakpoint (Pflugfelder *et al.*, 1990).

YAC technology. The following YACs and their noted polytene location were sent courtesy of Dr. Ian Duncan: DY-063 (3F1-4A5, 140 kb), DYR07-40 (3F1-4A3, 250 kb), DYN12-95 (3F4-4A5, 145 kb), and DYN16-77 (3F4-4A2, 150 kb). Five-milliliter cultures of yeast harboring YACs were grown in AHC selective (-Ura, -Trp) media (24 hr, 30°C). High-molecular-weight DNA was prepared in agar blocks (Carle *et al.*, 1991).

YAC analysis was performed on a CHEF gel electrophoresis system (Bio-Rad CHEF DRII). Agarose gels (1–1.5%) were run in 0.5× TBE (ramped pulse time 50 to 90 sec, 200 V, 24 hr). YAC DYR07 was purified in 1.5% low melting agarose gel (Sea Plaque, FMC) with a CHEF apparatus (10 to 30 sec ramped pulse time, 200 V, 40 hr), essentially as described by Couto *et al.* (1989).

Cloning of the 3F–4B1 region. We initiated chromosome walks in the 3F–4A region (Fig. 4a) using two entry points. We obtained

cosmid 57 from the *Drosophila* genome project (Heraklion, Crete) which was reported to hybridize to region 3F of the X-chromosome. We confirmed its location at 3F by *in situ* hybridization (not shown), and used it to identify YAC clone DYR07, which overlapped with the proximal end of c57 (Fig. 4a). *In situ* hybridization of DYR07 DNA confirmed that the 250-kb insert extended across the 4A region into polytene band 4B1. DNA from both c57 and YAC DYR07 was used to isolate overlapping cosmid clones in the 3F–4A region.

For a second entry into the 4A region we isolated a 17-kb *Xho*I genomic breakpoint fragment (λrb46JN) spanning the breakpoint of $Df(1)rb^{46}$. $Df(1)rb^{46}$, which complements *brn* (Banga *et al.*, 1986; Goode *et al.*, 1992), was originally reported to delete polytene chromosome bands 4A3-6 to 4C6-7. We determined by restriction mapping and *in situ* hybridization that the λrb46JN clone contained 12 kb of DNA adjacent to the rb^{46} deficiency break from polytene band 4B1, and that this clone did not overlap YAC DYR07. We used this 12-kb fragment (JN4B) to isolate overlapping cosmid and λ clones which extended our walk 10-kb proximal and 40-kb distal to the $Df(1)rb^{46}$ breakpoint (Fig. 4a). The distal clones were shown to overlap YAC DYR07, confirming we had isolated contiguous DNA clones spanning the 3F–4B1 region.

Localization of cho to the distal (3F) region of the walk. To facilitate the localization of *brn*, we isolated deficiencies which remove the visible eye color mutation *cho* (Goode, 1994). *cho* maps at 5.5, within the 3F region of the X-chromosome (Lindsley and Zimm, 1992). One of the *cho* mutations we isolated, cho^{L1}, complements not only *brn* (Fig. 4a), but also the two loci proximal and distal to *cho*, namely *echinus* and *male diplolethal* (*mdl*). This suggested that cho^{L1} is a deficiency with breakpoints located within our genomic walk. Using restriction fragments from cosmid clones as probes on genomic Southern blots, we localized the proximal and distal breakpoints of cho^{L1} to the 3F region (Fig. 4a). Additionally, we localized the distal breakpoint of $Df(1)rb^{1}$, which complements *cho* but not *mdl* and *brn*, to the same region (Fig. 4a). This placed *cho* in 40 kb of DNA at the distal end of our walk, between coordinates 50–90 kb.

RNA injections. mRNA was injected into preblastoderm embryos as described in the text and in Schneider *et al.* (1991).

Sequence analysis. A Sequenase 2.0 kit (U.S. Biochemical) was used for all sequencing reactions. The complete *brainiac* sequence was obtained on both strands using custom primers and universal primers located at the 5′ and 3′ ends of the pNB40 vector (Fig. 4). Standard sequencing reagents and equipment was used.

MacVector was used for sequence analysis. GenBank, EMBL, PIR-protein, PIR-nucleic, and Swiss protein and nucleic acid data bases were searched for proteins and nucleic acids with homology to the predicted *brainiac* amino acid and nucleotide sequences using FASTA and BLAST.

RESULTS

brn and grk Cooperativity during Follicular Morphogenesis

We have previously shown that *brn* is active in the germline and cooperates with the Egfr, expressed in the follicle cells, to establish the dorsal–ventral pattern of the follicular epithelium (Goode *et al.*, 1992). The TGFα-like product of the *grk* locus is expressed in the germline and is the presumptive ligand for the Egfr for dorsal–ventral patterning of the follicular epithelium (Neuman-Silberberg and Schüpbach, 1994; Roth *et al.*, 1995; González-Reyes *et al.*, 1995). Loss of *brn* function causes a ventralized eggshell phenotype similar to partial loss of *grk* TGFα or *top* Egfr function, in which the two dorsolaterally positioned respiratory appendages of the wild-type eggshell are fused along the dorsal midline of mutant eggshells (Figs. 1a–1c; Table 1).

To determine if *grk* activity depends on the function of the putative secreted product encoded by *brn* (see below), we tested for dosage-sensitive interactions between these genes. We found that eggs from animals heterozygous for weak mutations in either gene are completely wildtype ($brn^{fs.107}$/+ or grk^{ed22}/+), while animals doubly heterozygous for weak *brn* and *grk* mutations ($brn^{fs.107}$/+;grk^{ed22}/+) lay eggs with partially fused dorsal appendages (Fig. 1d; Table 1). Likewise, while eggs from animals heterozygous for strong alleles of either gene ($brn^{1.6P6}$/+ or grk^{HK36}/+) show slight haploinsufficiency for dorsal–ventral patterning of the follicular epithelium (Table 1), doubly heterozygous animals ($brn^{fs.107}$/+;grk^{HK36}/+ or $brn^{1.6P6}$/+;grk^{HK36}/+) show greater than additive, synergistic, defects in egg shell patterning (Fig. 1e; Table 1). To directly determine if *grk* cooperates with *brn* in eggshell patterning, we constructed $brn^{fs.107}$;grk^{ed22} double-mutant animals. While animals homozygous for either mutation alone lay eggs with fused dorsal appendages (Figs. 1b and 1c), $brn^{fs.107}$;grk^{ed22} double-mutant animals lay completely ventralized eggs, completely lacking dorsal appendages; these chorions resemble eggshells from grk^{HK36} animals, which are completely void of *grk* function (Figs. 1f and 1g; we cannot analyze the embryonic phenotype from double-mutant females since completely ventralized eggs are rarely fertilized and because these females lay only a few eggs). Our results indicate that the *brn* and *grk* gene products, presumably secreted from the germline, cooperate in dorsal–ventral patterning the follicular epithelium.

We also sought to determine if *brn* and *grk* cooperate in other aspects of follicular patterning. *grk* expression is initiated in the germline in region 2 of the germarium (Neuman-Silberberg and Schüpbach, 1993). This coincides with the time of *brn* expression (see below). We therefore asked whether *grk* is also required for the formation of the follicular epithelium by analyzing ovarian phenotypes in *grk* mutant ovaries and by testing for genetic interactions between *brn* and *grk* mutations. Ovarioles from females homozygous for either a weak *grk* allele, grk^{ed22}, or a weak *brn* allele, $brn^{fs.107}$, resemble wild type with each nurse cell–oocyte complex completely surrounded by a follicular epithelium (Table 1, Fig. 2). In contrast, egg chambers from animals homozygous for a strong *grk* allele, grk^{HK36}, or heterozygous for two strong *grk* alleles, grk^{HG21}/grk^{DC29}, consistently display both fused chambers and gaps (see next section) in the follicular epithelium (Table 1). Likewise, over half of egg chambers from $brn^{fs.107}$;grk^{ed22} females (doubly mutant for weak *brn* and *grk* alleles), display large gaps in the follicular epithelium, frequently uncovering over half of the egg chamber (Fig. 2; Table 1). We conclude that *grk* acts in concert with *brn* to achieve the migration of prefollicular

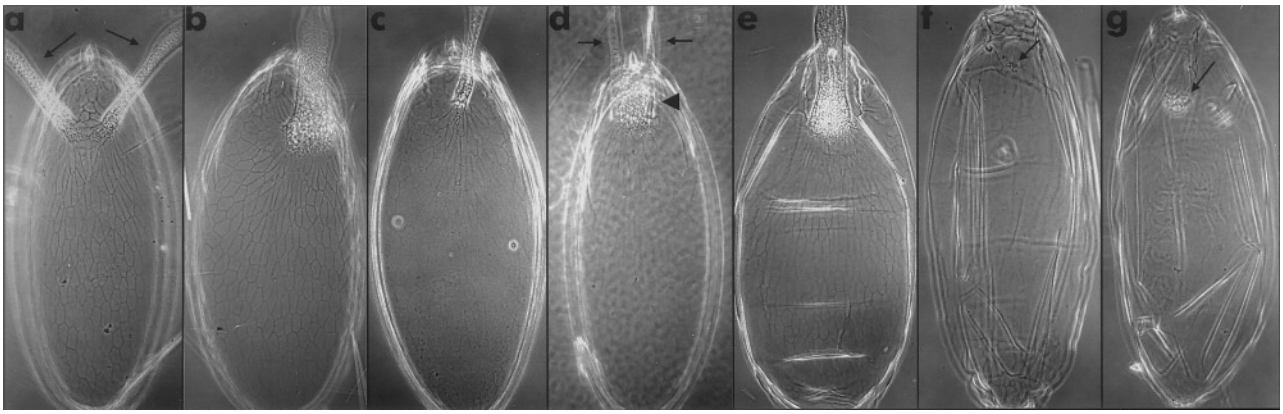

FIG. 1. *brn* and *grk* cooperate in eggshell patterning. The figure shows Hoyer's mounts of the dorsal surface of eggshells. (a) A wild-type egg. The arrows point to the dorsal appendages, which are located on the dorsal–lateral surface of the eggshell, and are derived from dorsal–lateral follicle cells. (b) An egg from a $brn^{fs.107}$ female. The dorsal appendages are fused along the dorsal midline, indicating that dorsal–lateral follicle cells are shifted to a more dorsal position. The dorsal appendage is about twice as wide as for a single dorsal appendage from a wild-type female. This is the most severe eggshell phenotype observed for *brn* mothers. (c) An egg from a grk^{ed22} female. The fused dorsal appendage is narrower than a single wild-type appendage (a), or the fused *brn* appendage (b). The significance of this difference is not clear. (d) An egg from a $brn^{fs.107}/+;grk^{ed22}/+$ female. The dorsal appendages are fused at the base (arrowhead), with two appendages readily apparent (arrows). This weak fused dorsal appendage phenotype is indistinguishable from the weak eggshell phenotype of eggs laid by homozygous *brn* females. (e) An egg from a $brn^{1.6P6}/+;grk^{HK36}/+$ female showing an intermediate to strong fused dorsal appendage phenotype that is indistinguishable from the intermediate to strong phenotype of some eggs laid by *brn* females. (f) A completely ventralized egg from a grk^{HK36} female. This is the most severe *grk* phenotype. The eggshell completely lacks dorsal appendages. The arrow points to a small fragment of appendage material. (g) A completely ventralized egg from a $grk^{ed22};brn^{fs.107}$ female. The arrow points to a small fragment of appendage material. The amount of appendage material remaining on eggs from grk^{HK36} or $grk^{ed22};brn^{fs.107}$ female is variable, and the greater amount shown in g is not reflective of the relative strength of the grk^{HK36} and $grk^{ed22};brn^{fs.107}$ eggshell phenotypes.

cells to surround each nurse cell–oocyte complex and to form a continuous epithelium.

Gaps in the Follicular Epithelium

As soon as mutant chambers separate from the germarium, gaps in the follicular epithelium are detected. Because these gaps are present as soon as chambers form, we assume that prefollicular cells have failed to migrate over the surface of all germ cells of a nurse cell–oocyte cyst in a manner similar to that previously shown for $brn^{fs.107}$; *top* chambers (Goode *et al.,* 1992). To determine if there is a bias in the location of follicular discontinuities, we examined individual egg chambers of different genotypes for gaps in the follicular epithelium. Egg chambers were stained with phalloidin to reveal the actin cortices of germ cells and follicle cells (Figs. 2 and 3). As shown in Table 1, 284 stage 2 to 7 egg chambers from animals mutant for various $Egfr^{t}$ (*torpedo* alleles, cf. Schüpbach, 1987), *grk*, and *brn* allele combinations were analyzed, and a gap in the follicular epithelium was never observed over the oocyte. We also characterized the approximate position of gaps along the anterior–posterior axis of the ovarian follicle. Any nurse cell, whether anterior, central, or immediately adjacent to the oocyte (Fig. 3), may not be covered by the follicular epithelium. The most frequent gaps are found in the central regions of the ovarian follicle (Table 1). These results suggest that *grk*/*brn* signals are not produced equally by all members of the nurse cell–oocyte cluster, or are differentially required by follicle cells covering certain members of the nurse cell–oocyte cluster.

Since it is known that at stage 9 of oogenesis almost all follicle cells covering nurse cells migrate to surround the oocyte, leaving only a few thin squamous follicle cells covering the nurse cells, we sought to determine if gaps observed at the light microscope level might be due to a precocious thinning of follicle cells. We undertook a detailed ultrastructural analysis of ovaries from $brn^{fs.107};Egfr^{t}$ females to further characterize these gaps. As shown in Fig. 3, nurse cells are in direct contact with the basal lamina of the ovariole sheath, thus clearly documenting that the gaps are true discontinuities of the follicular epithelium. These discontinuities are already visible in stage 2 follicles as they emerge from the germarium. Ultrastructural observations of follicles in region 2B of the germarium (cf. Mahowald and Strassheim, 1970, for a description of regions) indicate that wild-type cystocytes are occasionally in contact with the basal lamina. This suggests that the complete envelopment of follicles requires the migration of prefollicle cells around each NC–oocyte complex. Gaps are produced when FCs do not surround each nurse cell.

RFLP Mapping of brn

Previous cytogenetic and complementation analysis had localized *brn* to the 3F–4A region of the X-chromosome

TABLE 1

Penetrance of the Follicular Phenotypes of *top*, *grk*, *brn*, and *brn;grk* Mutant Females Used in This Study

Genotype[d]	% Eggs ventralized[a]			% Egg chambers[b]		% Epithelial discontinuities uncovering[c]			
	Weak	Intermediate	Strong	With epithelial discontinuities	That are compound	Nurse cells in anterior regions of the follicle	Nurse cells in central regions of the follicle	Nurse cells adjacent to the oocyte	The oocyte
$brn^{fs.107}/+$	0	0	0 (n = 822)	—[e]	—	—	—	—	—
$brn^{l.6P6}/+$	5	0	0 (n = 527)	—	—	—	—	—	—
$grk^{ed22}/+$	0	0	0 (n = 425)	—	—	—	—	—	—
$grk^{HK36}/+$	27	0	0 (n = 406)	—	—	—	—	—	—
$brn^{fs.107}/+;grk^{ed22}/+$	42	0	0 (n = 642)	—	—	—	—	—	—
$brn^{l.6P6}/+;grk^{ed22}/+$	53	0	0 (n = 432)	—	—	—	—	—	—
$brn^{fs.107}/+;grk^{HK36}/+$	71	0	0 (n = 406)	—	—	—	—	—	—
$brn^{.l.6P6}/+;grk^{HK36}/+$	78	0	0 (n = 433)	—	—	—	—	—	—
$brn^{fs.107}$	100	0	0 (n = 538)	0	2	—	—	—	—
grk^{ed22}	100	0	0 (n > 500)	0	<1 (n > 500)	—	—	—	—
grk^{HK36}	0	2	98 (n = 155)	20	15 (n = 190)	—	—	—	—
grk^{DC29}/grk^{HG21}	0	8	92 (n = 215)	18	14 (n = 153)	17	77	7	0 (n = 30)
$brn^{fs.107};grk^{ed22}$	0	27	73 (n = 196)	51	10 (n = 165)	13	67	19	0 (n = 89)
top^{1}/top^{IK35}	65	35	0 (n = 254)	2	<1 (n = 360)	36	60	12	0 (n = 50)
top^{1}/top^{IP02}	88	12	0 (n = 207)	2	<1 (n = 186)	42	50	8	0 (n = 20)
top^{1}/top^{2X51}	70	30	0 (n = 205)	2	<1 (n = 430)	29	40	31	0 (n = 30)
top^{CJ}/top^{IK35}	72	28	0 (n = 307)	2	<1 (n = 265)	40	58	21	0 (n = 65)

[a] Weak, intermediate, and strong designations are as described in Schüpbach (1987) and Goode *et al.* (1992).

[b] Epithelial discontinuities are as described in this study (see text and Figs. 1 and 2) and in Goode *et al.* (1992).

[c] See Fig. 3 for a description of the topology of epithelial discontinuities.

[d] For all heterozygous and doubly heterozygous combinations to wild-type chromosomes, only the eggshell pattern is disrupted. The embryos which develop from these females are completely wild-type.

[e] Dashes indicate that the category is not applicable. The *top* mutations are female sterile alleles of *Egfr*, and are designated $Egfr^{t}$ in the text.

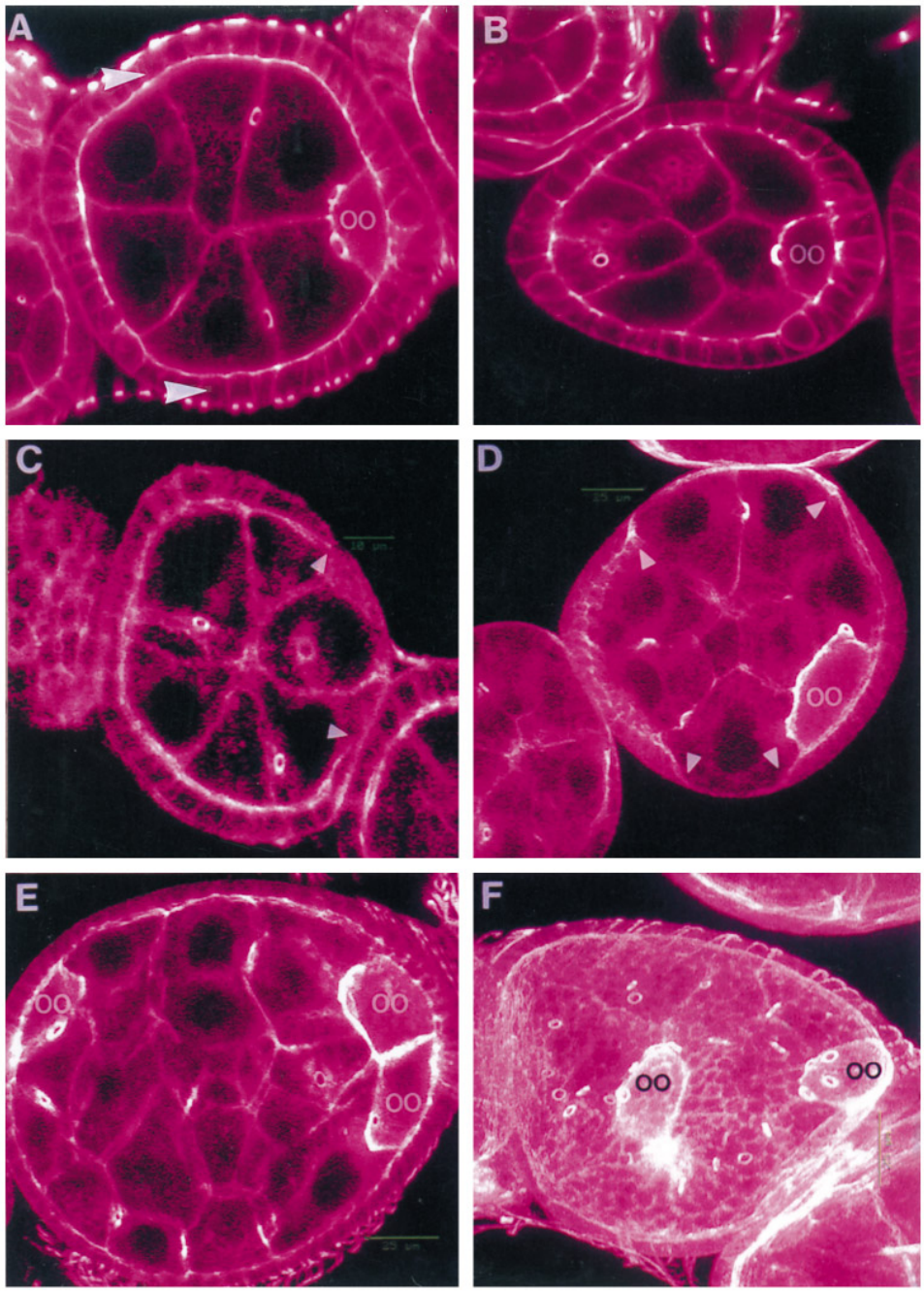

FIG. 2. *brn* cooperates with *grk* in follicular morphogenesis. Egg chambers from females of the indicated genotypes stained with phalloidin to reveal actin at cellular cortices. (A, B) Stage 4 to 5 egg chambers from females homozygous for the weak hypomorphic *grk* allele grk^{ed22} (A) or the hypomorphic *brn* allele $brn^{fs.107}$ (B) appear identical to wild type. grk^{ed22} and $brn^{fs.107}$ females lay eggs with weakly ventralized egg shells. The oocyte (oo) is always localized at the posterior of the egg chamber and is more enriched for actin and thus fluoresces more intensely than the nurse cells. The germ cells are surrounded by a monolayer epithelium of follicle cells (arrowheads). Note the bands of muscle surrounding the egg chambers, visible as extremely bright spots. Egg chambers from genetic null grk^{HK36} (C) and $brn^{fs.107};grk^{ed22}$ (D) females frequently have epithelial discontinuities (arrowheads). Additionally, egg chambers from grk^{HK36} and $brn^{fs.107};grk^{ed22}$ females often contain more than one oocyte–nurse cell complex (E, F). (E) Egg chamber from a $brn^{fs.107};grk^{ed22}$ female with three oocytes (oo), two at the posterior of the egg chamber and one at the anterior. This phenotype resembles the germ line clone phenotype of the strong *brn* allele $brn^{l.6P6}$ (Goode *et al.*, 1992). There are no ring canals between the two oocytes at the posterior of the egg chamber, indicating that they were not derived from the same blast cell, but represent the oocytes of two distinct oocyte–nurse cell complexes. Supernumerary

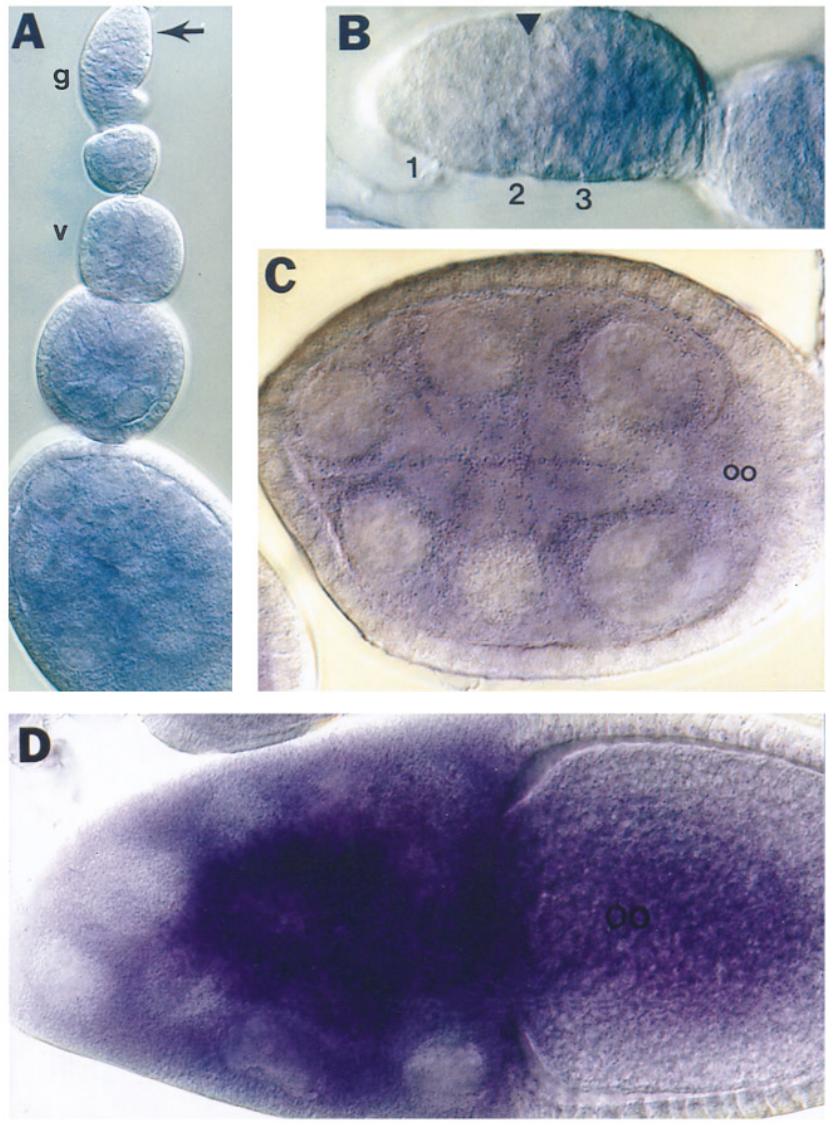

FIG. 8. *In situ* hybridization of *brn* transcript to wild-type ovarian tissue. Ovarian tissue from wild-type females was stained with antisense digoxigenin RNA probe. (A) Early egg chambers in the vitellarium (v) show uniform expression of *brn* in germ cells, as is probably the case when *brn* expression initiates in the germarium (arrow, g). *brn* does not appear to be expressed in follicle cells. Analysis of many stained germaria indicates that expression is not found along the periphery of the germarium, where follicle cell bodies are located. *brn* does not appear to be expressed in the most anterior regions of the germarium (arrow in A; region 1 in B), where germ cell divisions occur. (B) *brn* transcription appears to begin in region 2 (arrowhead), at the time that the cyst becomes surrounded by a monolayer of follicle cells. (C) A stage 6 egg chamber showing uniform levels of expression in nurse cells as well as the oocyte (oo). (D) At stage 10 of oogenesis *brn* transcript becomes very abundant in nurse cells, probably reflecting the maternal contribution of *brn* transcripts to early neurogenesis (Goode *et al.*, 1992). The *brn* message is rare, requiring unusually long exposures (6 to 8 hr). Apparent staining in follicle cells in B and C is due to light diffraction in images captured at higher magnifications; control, sense-strand probes give this same follicle cell background staining when exposed for 6 hr (not shown).

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oocyte–nurse cell complexes result from a failure of follicle cells to migrate and surround individual cysts during the early phases of oogenesis (Goode *et al.*, 1992). Supernumerary oocytes are typically found at the anterior or posterior poles of the egg chamber, suggesting an affinity of the oocyte for follicle cells at the anterior and posterior poles (E). Less frequently, oocytes are found at central positions within the egg chamber, as in (F). Even in these instances, the oocyte always contacts follicle cells, as shown by this confocal image taken in the plane of the follicular epithelium.

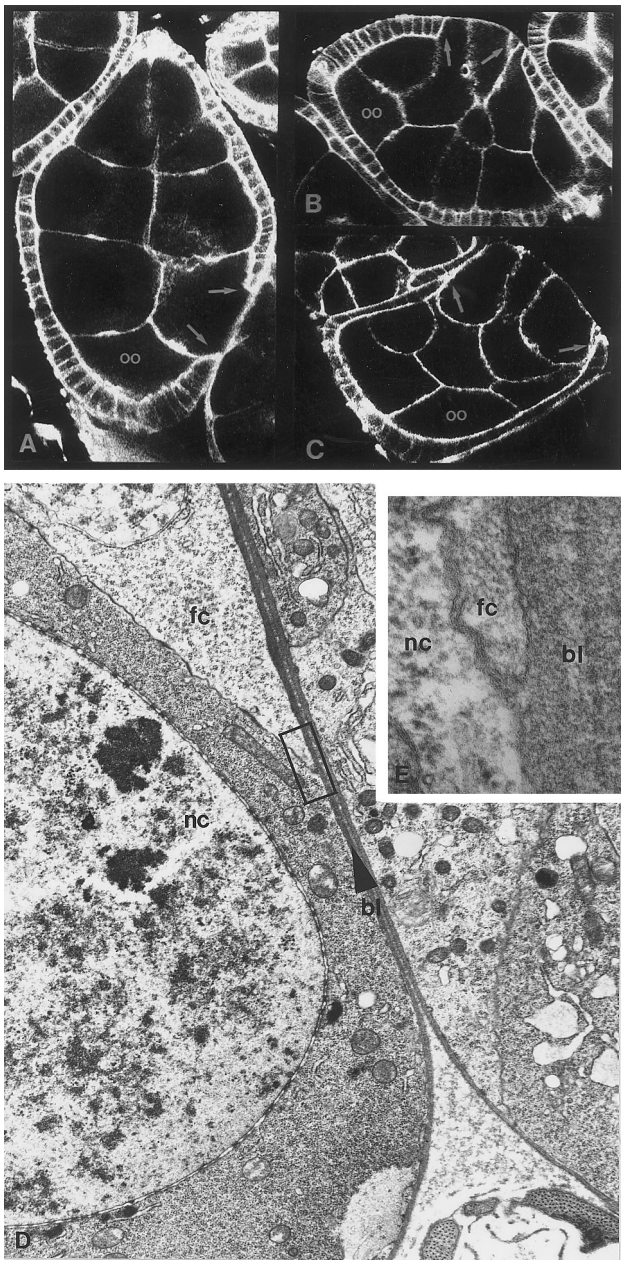

FIG. 3. Follicular discontinuities. Stage 6 to 7 ovarian follicles stained with phalloidin to reveal the actin cortex of germ and follicle cells. (A, B, C) Ovarian follicles from top^{2X51}/top^{1} females. We observe discontinuities only in the follicular epithelium covering nurse cells (arrows, see Table 1), but not over the oocyte (oo). Epithelial discontinuities are observed (A) in posterior regions, abutting the oocyte, (B) in central regions, or (C) in anterior regions of the follicle. (D) Electron micrograph of an epithelial discontinuity in a stage 5 egg chamber from a $brn^{fs.107}$;$Egfr^{t}$ female. The nurse cell (nc) comes into direct contact with the basal lamina (bl) of the chamber. The boxed area is shown at higher magnification in E.

(Goode *et al.*, 1992). We isolated 300 kb of genomic DNA spanning this region from yeast artificial chromosome (YAC), cosmid (c), and lambda (λ) libraries (described under Materials and Methods and summarized in Fig. 4a). *brn* had been previously mapped 0.4 ± 0.1 units proximal to *cho* (Goode *et al.*, 1992). Based on the known position of *cho* (Fig. 4a, Materials and Methods) and an estimate of 0.1 map units = ~40 kb DNA, we postulated that *brn* would reside within 40 kb of DNA at the proximal (4B) end of our walk (λJN region, Fig. 4a). To test this hypothesis, we designed and executed an RFLP mapping experiment between *cho* and *brn*.

Recombinant F_1 $cho^{+}-brn^{+}$ males were isolated from $cho^{2}brn^{+}/cho^{+}brn^{1.6P6}$ females and analyzed to determine their haplotype for the four RFLPs identified in the region. As summarized in Fig. 4b, we found that 6 of the 43 $cho^{+}-brn^{+}$ recombinant lines had the $brn^{1.6P6}$ haplotype for *Hpa*I, the most distal RFLP in the region, confirming that the $brn^{1.6P6}$ mutation maps proximal to the polymorphic *Hpa*I site, within the proximal 40 kb of our walk. Of these six lines, three show a *Sca*I polymorphism, two showed a *Sma*I polymorphism, and a single recombinant line had the $brn^{1.6P6}$ haplotype for the most proximal RFLP identified by *Xba*I. This indicated that the recombination event took place proximal to the *Xba*I site, and therefore that the $brn^{1.6P6}$ mutation must map proximal to this position, within 10 kb of the $Df(1)rb^{46}$ breakpoint (Fig. 4b).

Transcription Mapping of the brn Region

Since *brn* is a maternal effect gene expected to be expressed in early embryos, we characterized the embryonic transcripts expressed in the 10-kb *brn* region (Fig. 5). At least four distinct sets (A–D) of embryonic transcripts are expressed in this region. Two of these transcripts can be excluded as candidates for *brn*. Since transcript A is expressed in 4- to 8- and 8- to 12-hr but not 0- to 4-hr embryos, it appears to be a zygotically activated, rather than a maternal effect gene. This transcript also overlaps the *Xba*I polymorphism, indicating that *brn* lies proximal to this transcript. Transcript D is not a candidate for *brn* because the 5′ end of the gene is deleted by $Df(1)rb^{46}$, and this deficiency chromosome complements *brn*. Genes B and C remained as strong candidates for the *brn* transcription unit.

Gene B encodes a 1.3-kb mRNA which is constitutively expressed in the first 12 hr of embryogenesis (Fig. 6). We isolated several cDNAs from both ovary and embryonic libraries that appeared to be identical and full length. This mRNA appears to be rare based on its relative abundance on Northern blots (Fig. 6), the presence of only 3 cDNAs of over 300,000 screened, and the relatively long exposure time required for ovarian *in situ* hybridizations (see below). It should be noted that most of the RNA detected in Northern blots of ovaries is due to late ovarian stages (cf. Fig. 8).

Gene C encodes mRNAs of 0.95 and 1.25 kb which are expressed in 0- to 4- and 8- to 12-hr embryos. We isolated six different clones from a 0- to 4-hr embryonic cDNA library and these cDNAs spread over 5 kb of genomic DNA

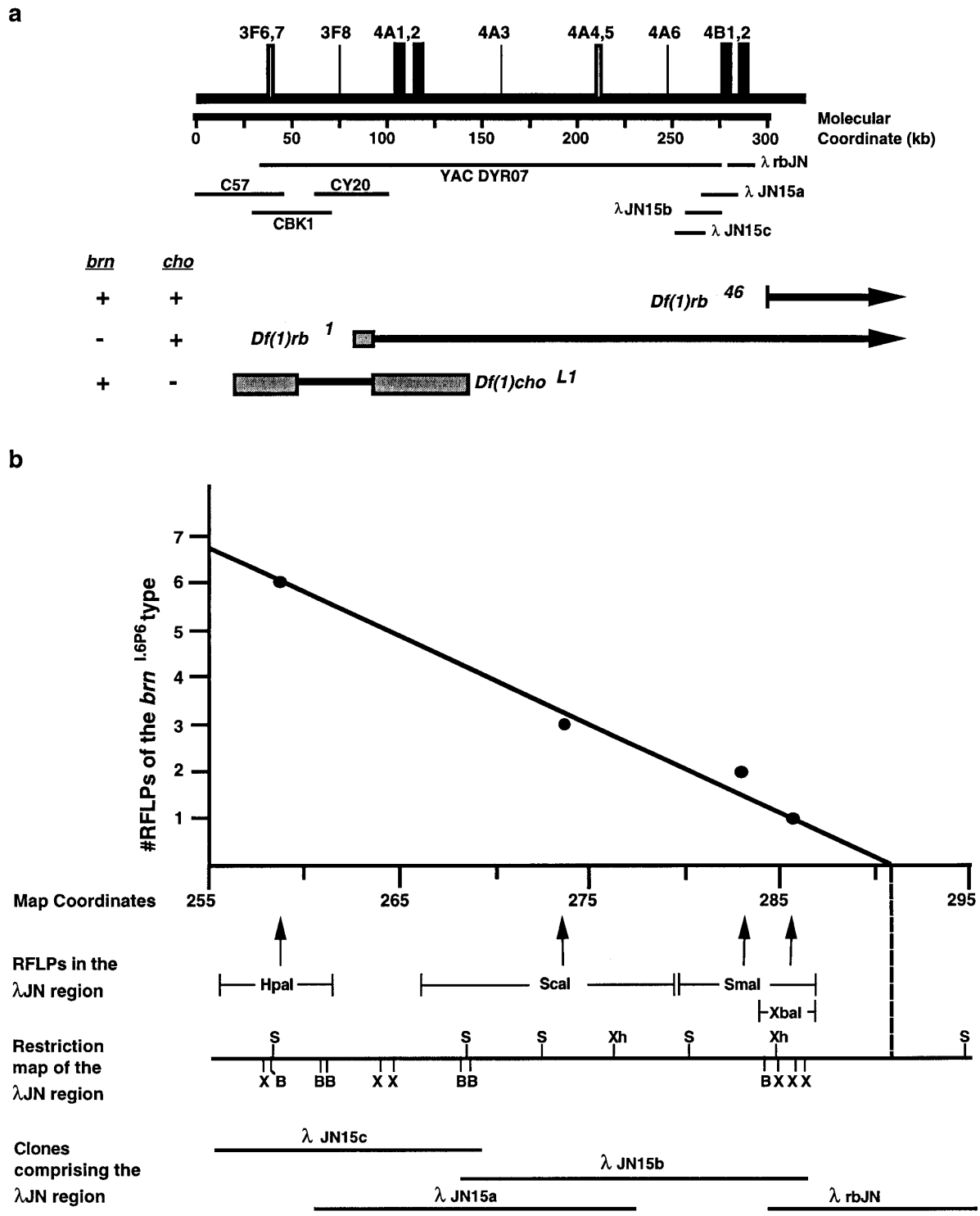

FIG. 4. Localization of *brainiac* to 10 kb of DNA. (a) Genomic walk and deficiency breakpoint analysis in the *brn* region. The top of the figure is a schematic representation of the 3F-4B chromosomal region to which *brn* had previously been localized (Goode *et al.*, 1992). Directly below, the molecular coordinates for the genomic walk are depicted, along with the positions of an overlapping yeast artificial chromosome (YAC) and cosmid (C) and lambda (λ) clones across the 3F–4B region. As shown below these DNA clones, both *cho* and *brn* were localized at molecular resolution by combined complementation (+ indicates complementation by the deficiency; – indicates failure to complement) and deficiency breakpoint analysis (solid bars indicate DNA deleted from the region, and shaded bars indicate the

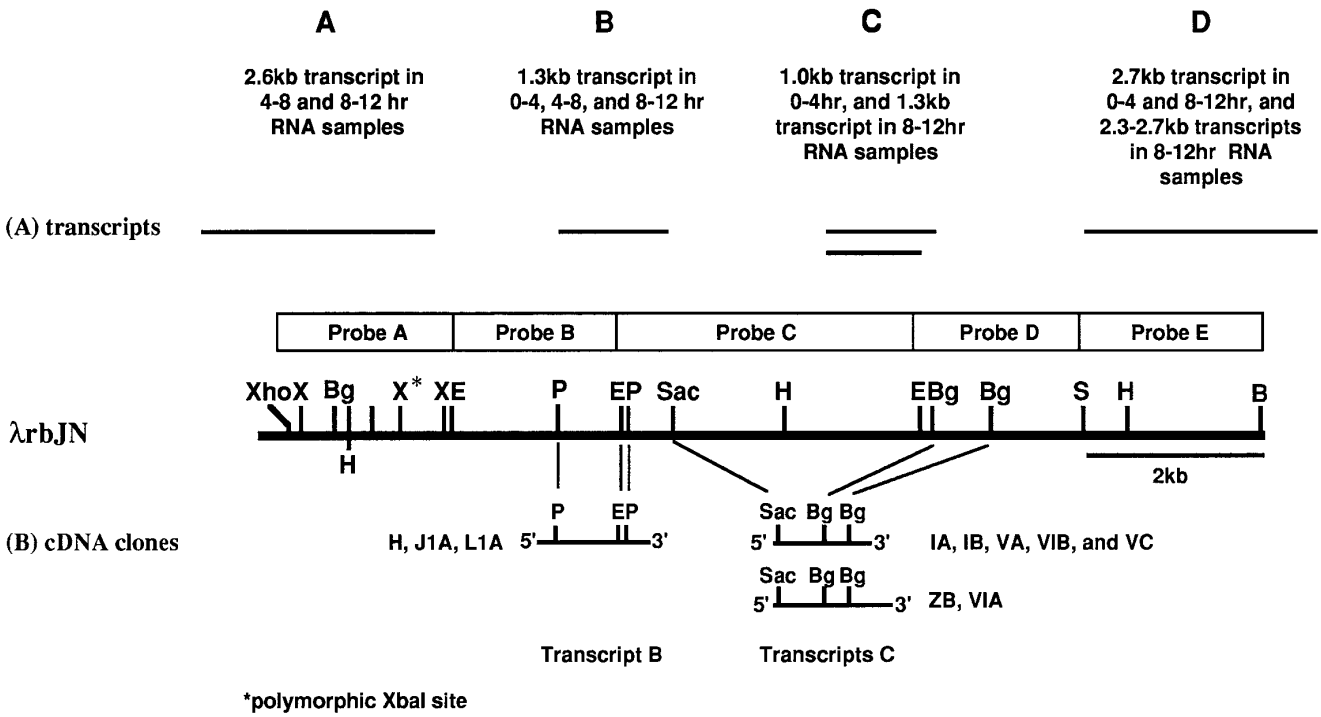

FIG. 5. Candidate *brn* transcription units. (A) Results of Northern analysis for the 10 kb of DNA containing *brn* (Fig. 4). The indicated *Xba*I polymorphism is the same as in Fig. 4. Northern blots of mRNA from 0- to 4-, 4- to 8-, and 8- to 12-hr embryos were analyzed with probes A–E, shown above the restriction map of λrbJN, and the results are summarized. If the line representing a transcript and the box representing a probe overlap, then the probe detects the transcript(s) (data not shown). As described in the text, transcripts A and D were ruled out as candidate *brn* transcription units. (B) Restriction sites and relative positions of cDNAs corresponding to the two candidate *brn* transcription units are shown. The direction of transcription for both transcription units were established as described under Materials and Methods. The sequences of cDNAs IA, IB, VA, VC, VIA, VIB, and ZB appear to spread over 5 kb of genomic sequences (as shown), suggesting that the gene products result from alternative splicing of exons encoded within 5 kb of genomic DNA. Sequence analysis (Goode, 1994) is consistent with several alternatively spliced transcripts being encoded by this locus. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sac, *Sac*II; X, *Xba*I; Xho, *Xho*I.

(cf. Fig. 5), suggesting that they may arise from alternative splicing events.

RNA Rescue of brn Embryos

Since the maternal effect, neurogenic phenotype of embryos from *brn* eggs can be zygotically rescued by fertilization with brn^+-carrying sperm (Perrimon *et al.*, 1989; Goode *et al.*, 1992), we designed an RNA rescue experiment to test the B and C genes for *brn* function. We synthesized mRNA *in vitro* from cDNA clones which appeared to contain complete coding regions based on our restriction mapping and preliminary sequence analysis. For gene B we used J1A, a 1.3-kb cDNA clone which was isolated from a 4- to 8-hr embryonic cDNA library (Brown and Kafatos, 1988) and whose sequence appeared to contain the whole gene (see below). To test gene C, we synthesized mRNA from several cDNA templates

uncertainty in this analysis; see Materials and Methods for details). Based on this analysis, *cho* was localized approximately between coordinates 50 and 90, while *brn* was localized between coordinates 90 and 295. As described in the text, we used these findings to postulate that *brn* resided within 40 kb of DNA at the proximal end of our walk. (b) Restriction fragment length polymorphisms (RFLPs) were used to localize *brn* to 10 kb of DNA. Clones in the λJN region were used to map RFLPs between a cho^2 chromosome and a $brn^{l.6P6}$ chromosome at the proximal end of the genomic walk. Four RFLPs (*Hpa*I, *Sca*I, *Sma*I, and *Xba*I) were chosen for analysis; the degree of uncertainty in the position of the RFLP is indicated by a bar. As described by the graph, a strict linear relationship was observed in cho^+, brn^+ recombinants between the number of RFLPs of the $brn^{l.6P6}$ type versus position within the walk. A single *Xba*I RFLP of the $brn^{l.6P6}$ type, localized between coordinates 284–286 was identified, which combined with the position of the $Df(1)rb^{46}$ breakpoint (see above), decisively localized *brn* to the most proximal 10 kb of DNA within our walk. B, *Bam*HI; S, *Sal*I; X, *Xba*I; Xh, *Xho*I.

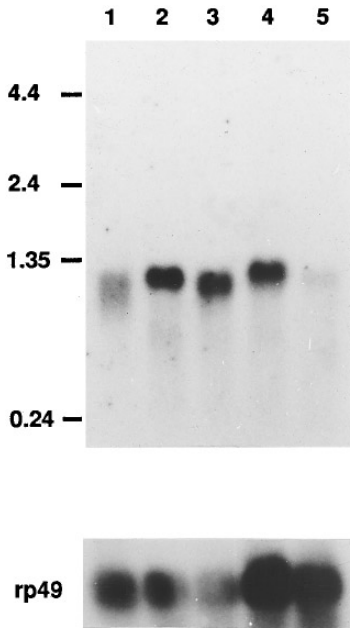

FIG. 6. Developmental Northern of *brn* transcripts. Northern blot of ovary and embryonic mRNAs hybridized with *brn* cDNA probe. The lanes are (1) ovary, (2) 0- to 4-hr embryos, (3) 4- to 8-hr embryos, (4) 8- to 12-hr embryos, (5) 12- to 20-hr embryos. Lanes 1–3 contain 1–2 μg poly(A)$^+$ RNA; lanes 4–5 contain 3–5 μg poly(A)$^+$ RNA. For comparison of the relative amounts of RNA in each lane, the filter was reprobed with the ribosomal RNA gene, *rp49*. A single mRNA of approximately 1.3 kb is present at all stages, with the highest expression during 0- to 4- and 4- to 8-hr stages of embryogenesis. The *brn* message appears to undergo slight size modifications during embryogenesis; sequencing of clones from ovary and different embryonic stage cDNA libraries indicates that these are due to differences in the length of the *brn* 3′ untranslated region.

(1A, IB, VA, VC, VIA, and ZB; see Fig. 5 and Materials and Methods) because our preliminary sequence analysis indicated that the coding region of each of these representative cDNAs differed in size.

The mRNAs were injected into 1- to 2-hr embryos derived from $brn^{l.6P6}/brn^{fs.107}$ females crossed to $brn^{fs.107}/Y$ males (these embryos never hatch and 99% display the most severe neurogenic phenotype) (Goode *et al.*, 1992). Of the *brn* embryos that had completed gastrulation following injection of transcription unit C transcripts, none hatched (0/62) and most (60/62) had the most severe *brn* neurogenic phenotype (Table 2). In contrast, when transcripts synthesized from cDNA J1A (corresponding to transcription unit B) were injected, 13 of 50 embryos hatched, and most unhatched embryos (30/37) had less severe phenotypes. These results indicated that transcription unit B encodes the *brn* gene product. *brn* appears to be expressed constitutively throughout embryogenesis (Fig. 6), and it is expressed in adult males as well as females (data not shown), consistent with *brn*'s requirement for early neurogenesis, adult viability, and oogenesis.

brn encodes a Novel, Putative Secreted Protein

Sequence analysis revealed no difference between cDNAs H, JIA, and genomic coding sequences, thus indicating that the *brn* gene has no intron sequences within the coding region (Fig. 7). Conceptual translation of the *brn* polypeptide (Fig. 7) and comparison to proteins in the GenBank, EMBL, PIR-protein, PIR-nucleic, and Swiss protein and nucleic acid data bases using the BLAST and FASTA programs revealed no similarity to known proteins. Hydropathy analysis of the predicted *brn* polypeptide revealed a single hydrophilic cluster of amino acids at the amino terminus of the protein (Fig. 7b), a common structural feature of secreted proteins. Indeed, amino acids 1 to 29 have several features that are characteristic of eukaryotic signal sequences, including the ordered classes of amino acids that are canonical for eukaryotic signal peptide cleavage sites (Fig. 7A; von Heijne, 1985). The putative *brn* polypeptide has two potential N-linked glycosylation sites and 25 serine and 11 threonine residues that may serve as sites for o-linked glycosylation (Fig. 7A).

brn Expression Initiates in Region 2 of the Germarium and Is Restricted to the Germline

To determine the expression pattern of *brn* during oogenesis, we hybridized *brn* sequences to ovarian tissue. As shown in Fig. 8A, *brn* expression is restricted to germ cells. Expression begins in region 2 of the germarium and continues throughout oogenesis at a low, uniform level in all germ cells until stage 10A. At stage 10A, *brn* mRNA accumulation increases dramatically in nurse cells and the oocyte (Fig. 8), probably reflecting the maternal requirement of *brn* for neurogenesis.

TABLE 2
Transcript B Encodes the *Brainiac* Gene Product

	No. of embryos injected	No rescue	Partial rescue	Complete rescue, without hatching	Hatching larvae
Transcript B	50	7	12	18	13
Transcript C	62	60	1	1	0

Note. mRNA corresponding to transcripts B and C (Fig. 5) was synthesized *in vitro*. Transcripts were injected (independently) into 0- to 2-h embryos from $brn^{l.6P6}/brn^{fs.107}$ mothers crossed to $brn^{fs.107}/Y$ males (Materials and Methods). Ninety-nine percent of uninjected embryos have no denticle belts when reared at 25°C. Embryos without denticle belts are indicated by ''no rescue'' in the table. ''Partial rescue'' indicates that at least one denticle belt was observed. ''Complete rescue, without hatching'' indicates that the embryos look essentially wild type (some embryos have defective cephalic cuticle), but do not hatch. ''Hatching larvae'' indicates that injected embryos developed and hatched as first instar larvae. Most embryos injected with transcript B either developed completely, or hatched, whereas most embryos injected with transcript C showed no signs of rescue. These results indicate that transcript B encodes the *brainiac* gene product.

A

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TTTATTTTGGTCGCCATGCAAAGTAAACACCGCAAACTGCTGCTGCGCTGCCTCCTGGTGCTGCCCCTCATCCTTCTGGTGGACTATTGCGGCCTGCTGA  100
                M  Q  S  K  H  R  K  L  L  L  R  C  L  L  V  L  P  L  I  L  L  V  D  Y  C  G  L  L    28

CCCACCTGCACGAGCTGAACTTCGAGCGGCACTTTCACTACCCGCTGAACGATGACACTGGATCTGGGTCCGCATCCTCTGGACTGGACAAGTTCGCCTA  200
T  H  L  H  E  L  N  F  E  R  H  F  H  Y  P  L  N  D  D  T  G  S  G  S  A  S  S  G  L  D  K  F  A    62

          <--------------------
CCTGCGAGTGCCCTCGTTCACGGCAGAGGTGCCGGTTGACCAGCCTGCGCGGCTCACGATGCTCATAAAGAGCGCGGTGGGCAATTCGCGACGTCGGGAG  300
Y  L  R  V  P  S  F  T  A  E  V  P  V  D  Q  P  A  R  L  T  M  L  I  K  S  A  V  G  N  S  R  R  R  E    95

GCCATTCGCCGGACTTGGGGCTACGAGGGTCGATTCTCAGACGTCCATCTGCGTCGCGTGTTCCTGCTGGGCACGGCCGAGGATAGTGAGAAAGACGTGG  400
  A  I  R  R  T  W  G  Y  E  G  R  F  S  D  V  H  L  R  R  V  F  L  L  G  T  A  E  D  S  E  K  D  V   128

                                                    ---------------------->
CTTGGGAGTCCCGCGAGCATGGCGACATTCTGCAGGCGGACTTCACGGACGCCTACTTCAACAACACGCTGAAAACCATGCTGGGTATGCGCTGGGCTAG  500
 A  W  E  S  R  E  H  G  D  I  L  Q  A  D  F  T  D  A  Y  F  N  N  T  L  K  T  M  L  G  M  R  W  A   162

                                  <----------------------
CGAGCAGTTCAATCGCAGCGAGTTCTATCTTTTCGTGGACGACGACTACTATGTTTCGGCCAAGAACGTGCTCAAGTTTCTGGGCAGGGGGCGTCAGAGC  600
S  E  Q  F  N  R  S  E  F  Y  L  F  V  D  D  D  Y  Y  V  S  A  K  N  V  L  K  F  L  G  R  G  R  Q  S  195

CACCAGCCGGAGTTGCTGTTCGCCGGGCACGTCTTTCAGACATCCCCGCTCCGCCACAAGTTCAGCAAGTGGTACGTTTCGCTGGAGGAATACCCCTTCG  700
  H  Q  P  E  L  L  F  A  G  H  V  F  Q  T  S  P  L  R  H  K  F  S  K  W  Y  V  S  L  E  E  Y  P  F   228

                          ---------------------->
ATCGATGGCCACCGTACGTGACCGCCGGCGCGTTCATTCTGTCGCAGAAGGCACTGCGCCAGCTGTATGCCGCCAGCGTTCACCTGCCGCTCTTCCGGTT  800
 D  R  W  P  P  Y  V  T  A  G  A  F  I  L  S  Q  K  A  L  R  Q  L  Y  A  A  S  V  H  L  P  L  F  R   262

                    <--------------------
CGACGACGTCTACCTGGGCATAGTGGCCTTGAAAGCGGGCATTTCCCTTCAGCACTGCGACGACTTCCGCTTCCATCGGCCGGCGTACAAAGGTCCGGAC  900
F  D  D  V  Y  L  G  I  V  A  L  K  A  G  I  S  L  Q  H  C  D  D  F  R  F  H  R  P  A  Y  K  G  P  D  295

AGCTATAGTTCGGTGATAGCCTCGCACGAATTCGGCGATCCCGAGGAGATGACGCGGGTATGGAACGAGTGCCGATCCGCCAATTACGCGTAGCAGCAAG 1000
  S  Y  S  S  V  I  A  S  H  E  F  G  D  P  E  E  M  T  R  V  W  N  E  C  R  S  A  N  Y  A  *          325

                                                                    <----------------
AACAGGCAGGACTGAAGAGCGCTGCAGATTGTACATATATACGAACGAGACAGCACCTAGACAACAGCCTTATAAAGTATATTAGGAGCAGTGGATGATA 1100
----->
CCTCAACCGTTCAGTGAGACTTTAAACCAAGCAGCTGGTGCTAATAGTGCAAGATCGCAAGAGTAATTCAATTCTAATTGTAAGCGTAATGATAAAGCAG 1200

ATGATTCAATTCGTATATATAGAGATTATGTGCAAATGTCGGATAACGTATGTGTGGTCTGATACCTTTGAATGCAATATAAAGTTGATTAACATAGGAA 1300

AGCGAATAAAAAACTTAAAATGAAAAAAAAAAAAACGAAT
        Genomic DNA: AAATGATTGGCTTGCATTT
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B

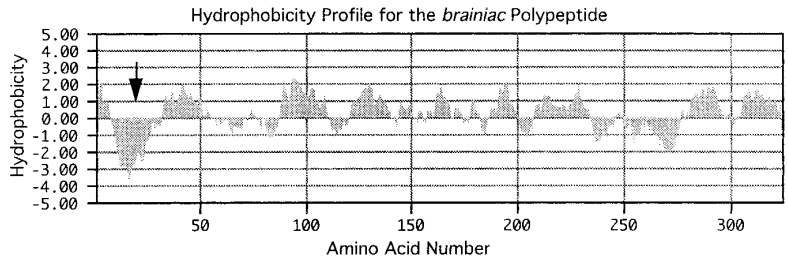

FIG. 7. Sequence and structural characteristics of the *brn* gene product. (A) Custom primers (indicated by dashed lines above the nucleotide sequence) and vector primers were used to obtain the complete sequence for *brn* genomic DNA and cDNA J1A. The sequences are identical, except at the carboxy terminus, suggesting that the coding regions for *brn* have no intron sequences. An ATG start codon and TAG stop codon are underlined, and an AATAAA polyadenylation signal is indicated in bold. Conceptual translation predicts a polypeptide of 325 amino acids. Comparison of *brn* nucleic acid and protein sequences in GenBank, EMBL, PIR-protein, PIR-nucleic, and Swiss protein and nucleic acid databases using the BLAST and FASTA programs revealed no similarity to known proteins. The *brn* sequence can be obtained under GenBank Accession No. U41449. (B) Hydropathy analysis indicates that the NH_4 terminus of the predicted *brainiac* protein is hydrophobic (arrow), a characteristic of signal sequences. Amino acids 1 to 29 (underlined in A) have several features that are characteristic of eukaryotic signal sequences, including the ordered classes of amino acids that are canonical for eukaryotic signal peptide cleavage sites (von Heijne, 1985). Cleavage of the 29-amino-acid signal peptide from the *brn* protein would produce a 296-amino-acid polypeptide. Without glycosylation, the molecular weight of the mature protein is expected to be 34 kDa. As expected for a secreted protein, the *brn* polypeptide has two potential N-linked glycosylation sites (NNT and NRS sequences highlighted in bold (Marshall, 1972)) and 25 serine and 11 threonine residues that might serve as sites for o-linked glycosylation.

DISCUSSION

brn Expression Pattern and Predicted Gene Product

Developmental genetic analysis of *brn* function indicates that *brn* is required continuously throughout oogenesis, beginning in the germarium at the time that follicle cells envelop the oocyte–nurse cell complex and continuing to stages when the eggshell is produced (Goode *et al.,* 1992). *brn* expression mirrors this requirement. *brn* does not appear to be expressed in region 1 of the germarium, where germ cell divisions essential for the formation of the germ cell cyst are accomplished. *brn* expression is initiated once the cyst has formed, in region 2a of the germarium, and is expressed in the germline continuously throughout oogenesis. This expression pattern is consistent with its role in developing the follicular epithelium around each germline cyst, as well as for dorsal–ventral patterning of the follicular epithelium during the later phases of oogenesis. Further, the finding that *brn* appears to encode a secreted protein is consistent with the nonautonomy and differential activity of *brn* in regulating follicle cell behaviors (Goode *et al.,* 1992). We discuss possible biochemical functions for *brn* below.

brn, grk, and Egfr Function in Follicular Morphogenesis and the Regulation of Distinct Cellular Phenotypes

The role of the Grk–Egfr signaling system in establishing both anterior–posterior and dorsal–ventral cell fates during oogenesis has recently been reviewed (Lehmann, 1995). In this and a previous study, we determined that the Grk–Egfr signaling system plays yet a third role to establish the follicular epithelium. The *grk* TGFα-like gene product acts as a ligand regulating a cascade of Egfr functions progressing through oogenesis (modeled in Goode *et al.,* 1992). These findings emphasize the problem of how the same signaling system can regulate distinct cellular properties among similar cells.

One observation that may be relevant to this problem is the finding that females harboring weak *brn, grk,* or *Egfr* mutations lay weakly ventralized eggs but have normal germaria capable of sustaining a wild-type rate of egg production (this study; Goode *et al.,* 1992). In contrast, flies harboring strong *brn, grk,* or *Egfr* mutations produce only a few eggs due to the formation of fused chambers and discontinuous epithelia, and in the case of *grk* and *top,* these eggs are completely ventralized. This finding is consistent with our previous hypothesis that the level of activity of *brn, grk,* and/or *Egfr* increase at stage 6 to 7 of oogenesis for dorsal–ventral patterning of the ovarian follicle (Goode *et al.,* 1992). Indeed, *grk* is known to be dosage sensitive (this study; Neuman-Silberberg and Schüpbach, 1994), and is expressed at increasingly higher levels throughout oogenesis: low at the time the follicle is formed in the germarium, intermediate in the oocyte during stages 1–7 when posterior cell fates are being specified, and high at stages 8–10 around the germinal vesicle when dorsal fates are being specified (Neuman-Silberberg and Schüpbach, 1993). These findings suggest that the level of activity of the TGFα–Egfr signaling system may play a fundamental role in imparting developmental specificity.

grk may not be the only TGFα-like molecule required for follicle production. We have found that the chorion phenotype of eggs laid by grk^{ed22} females is indistinguishable from the phenotype of eggs laid by $Egfr^{t}$ mutant females (this study). In contrast, the penetrance of egg chambers having supernumerary nurse cell–oocyte complexes, which results from a failure of prefollicle cells to initiate migrations into the germarium (Goode *et al.,* 1992), is much less in $brn^{fs.107}$;grk^{ed22} females (10%) than in $brn^{fs.107}$;$Egfr^{t}$ females (90%). Furthermore, we never observe egg chambers with more than 3 oocytes in $brn^{fs.107}$;grk^{ed22} females (this study), whereas egg chambers from $brn^{fs.107}$;$Egfr^{t}$ females can have as many as 6 to 7 oocytes (Goode *et al.,* 1992). In contrast, the penetrance of follicular discontinuities, a separate, distinct phenotype from the supernumerary nurse cell–oocyte complex phenotype (Goode *et al.,* 1992), is comparable in $brn^{fs.107}$;grk^{ed22} versus $brn^{fs.107}$;$Egfr^{t}$ females. These results lead us to speculate that *grk* is a critical ligand in regulating Egfr activity in the establishment of a complete follicular epithelium and for determining follicle cell fates, but that another ligand may play a critical role in regulating Egfr activity during prefollicle cell migrations. For example, one TGFα-like molecule may be generally expressed, and generally promote cell proliferation and migration, while a distinct TGFα-like molecule might be expressed with a distinct subcellular localization that specifically attracts follicle cell processes to a discreet destination on the germ cell cyst. Both TGFα and Egfr are known to be involved in regulating cellular migrations and epithelial development in vertebrate systems (Miettinen *et al.,* 1995; Reneker, 1995). We suggest that the spatial–temporal regulation of multiple Egfr ligands may play a role in establishing specificity of kinase signals. Putative Egfr ligands other than *grk* have been described for other phases of *Drosophila* development (Rutledge *et al.,* 1992).

A consideration of the *brn–grk* interaction at a molecular level suggests a third possibility for imparting specificity to Egfr signals. Grk belongs to the TGFα family of secreted proteins and we have found that *brn* acts nonautonomously (Goode *et al.,* 1992) and codes for a protein with a signal peptide, indicating that it probably is secreted. Our genetic data suggest that these factors cooperate to modulate follicle cell function. One possibility is that Brn directly regulates Grk activity by posttranslational modification or by regulating access or localization of Grk to the Egfr. This model predicts that in the complete absence of Grk activity, *brn* function should not be required (see Fig. 9). In contrast, we observe that the penetrance of discontinuities in $brn^{fs.107}$;grk^{ed22} animals, which contain sufficient *grk* activity for epithelial formation, is more than twice as great as that found for egg chambers from animals completely lacking *grk* function (grk^{HK36} animals; Table 1). These results

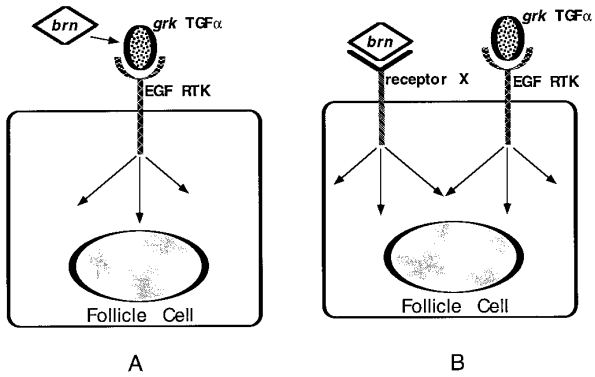

FIG. 9. Conceptual models for *brn* function during oogenesis. The models are suggestions to explain the synergistic interactions between the Brn and Grk molecules at a molecular level. (A) According to this model, Brn is needed for the full activity and/or localization of the Grk molecule, either directly or as part of a supermolecular structure. We do not favor this model because animals doubly mutant for weak *brn* and *grk* mutations, in which the activity of the Brn and Grk molecules are reduced, have much more severe phenotypes than animals completely lacking Grk molecule (see text), indicating that the Grk TGFα-like molecule does not directly depend on *brn* function. This explanation does not exclude the possibility that Brn might interact with, and regulate the activity of, several ligands. We also do not favor this model because *brn* and *grk* mutations give distinct phenotypes throughout most phases of the life cycle. (B) As described below and in the text, this model is most consistent with genetic data. Brn acts in a parallel pathway and binds to its own receptor, which may simply be a signaling receptor, or an adhesion receptor with signaling potential. In either case, the synergistic interactions between Brn and Grk may be explained by at least some of the signals from the receptors overlapping in their requirement for a common cellular behavior(s), as suggested by the arrows in the center of the cell. This explanation does not exclude the possibility that Brn might indirectly regulate the activity of several signaling systems, in addition to the Egfr-signaling system, due to a general failure in cell adhesion.

confirm that *brn* functions via an independent, parallel pathway to the inductive *grk* TGFα–Egfr signaling pathway, which is not surprising given the distinct phenotypes that are produced by mutations in these genes throughout most phases of the life cycle. Accordingly, we propose that Brn functions as a signaling molecule for a novel receptor (receptor X). Activation of receptor X stimulates intracellular signals which at least partially overlap with those generated by Grk binding to the Egfr (Fig. 9). Our model does not rule out the possibility that Brn acts in a parallel pathway to regulate adhesion between germ and follicle cells, which is also likely to involve overlapping signals between Egfr and the presumptive Brn receptor (Fig. 9). In either case, the action of *brn* in a parallel, but partially overlapping pathway to the Grk–Egfr signaling system, may play a crucial role in helping establish specificity to otherwise homogenous Egfr signals. Unidentified ligands and receptors have been proposed to regulate parallel, but partially overlapping pathways to receptor tyrosine kinases in vertebrate systems (Coso *et al.*, 1995).

Spatially Localized Gaps in the Follicular Epithelium

One explanation for the presence of gaps in the follicular epithelium is that follicle cells undergo premature thinning, similar to that occurring in follicle cells surrounding the nurse chamber during vitellogenesis. Our ultrastructural studies, however, have indicated that gaps are true discontinuities of the epithelium. Moreover, gaps already appear at the time that oocyte–nurse cell clusters form the germarium. This result suggests that gaps are caused by failure of follicle cells to migrate over and adhere to some of the nurse cells during early phases of follicular morphogenesis. We do not favor a reduction in follicle cell number as a contributing factor in the production of gaps, since this would be expected to produce a thinner epithelium, but clearly more investigation will be required to rule out this possibility.

For mutant egg chambers having an incomplete follicular epithelium, the oocyte is always covered by follicle cells, indicating that it has distinctive adhesive features from an early developmental stage. Several features have been shown to distinguish the oocyte from the nurse cells as soon as the follicle is formed in the germarium. Some gene products accumulate at high levels in one cell in region 2A of the germarium (*osk*, Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991; *orb*, Lantz *et al.*, 1992; *Bicaudal D*, Suter and Steward, 1991; *yl*, Schonbaum and Mahowald, unpublished observation), just prior to the time that nurse cell centrioles aggregate in the oocyte (Mahowald and Strassheim, 1970) and the formation of a polarized microtubule network (Theurkauf *et al.*, 1993). Some genes are expressed in follicle cells covering the oocyte, but not follicle cells covering nurse cells (Grossniklaus *et al.*, 1989), presumably in response to cues from the oocyte. Finally, the oocyte is enriched for actin compared to the nurse cells (Fig. 6). Based on our functional data, we propose that a differential follicle cell adhesive system exists for the oocyte relative to the nurse cells, which is likely to play a role in helping to orient the oocyte–nurse cell complex within the follicular epithelium during the early phases of oogenesis.

brn Neurogenic Function

Several observations suggest that *brn* is not required for Notch receptor function in lateral specification during oogenesis or early neurogenesis. N^{ts1}; $brn^{fs.107}$ double-mutant animals do not display ovarian phenotypes any more severe than either mutant alone; furthermore, a duplication of the *N* gene has no effect on the *brn* neurogenic phenotype (Goode, 1994), and *gooseberry* expression, a sensitive assay for maternal *Notch* activity, is not altered in *brn* mutant embryos (A. Manoukien and S. Goode, unpublished data). Additionally, *brn* does not appear to share the requirement of other neurogenic loci in regulating cell fate decisions requiring lateral specification in the pupal ectoderm (Goode, 1994).

brn is the first neurogenic gene found to encode a putative secreted protein, a biochemical function which has not been

described for any gene involved in lateral specification, but is found for several proteins involved in epithelial morphogenesis (Montesano *et al.*, 1991; Miettinen *et al.*, 1995). These results are consistent with the unique nature of *brn* germline activity among neurogenic genes. *brn* acts in the germline to regulate at least three follicle cell activities dependent on *Drosophila* Egfr and *grk* TGFα-like function (Goode *et al.*, 1992; this study). Thus, *brn* is a unique neurogenic gene that cooperates with the Egfr in the development and maintenance of epithelial structure. The morphogenesis of epithelial structure has been found to depend on tyrosine kinase function in vertebrates as well (Naldini *et al.*, 1991; Miettinen *et al.*, 1995).

Taking the biochemical nature and germline requirement for *brn* function as a clue, we propose that *brn* may not be required specifically for lateral inhibition during early neurogenesis, but rather is required in a parallel process needed to maintain epithelial structure within the neurogenic ectoderm during neuroblast segregation. Disruption of the continuity of the ectodermal epithelium may indirectly cause a neurogenic phenotype due to a compromised intercellular signaling capacity of neurectodermal cells. This proposal is consistent with the observation of Hartenstein *et al.* (1992) that neurogenic gene function is required for epithelial development, and suggests a specialized role for *brn* in epithelial maintenance.

ACKNOWLEDGMENTS

We thank John Perrino for excellent technical assistance. Ian Duncan kindly sent YACs. Some cosmids were a gift of the Crete Genome project. We gladly thank Peter Engler for help with YAC technology, and Trudi Schüpbach for contributing stocks and helpful information. Dan Gottschling provided a CHEF gel apparatus and Mark Garfinkel assisted in constructing the *Df(1)rb*^{46} genomic library. Thanks to Norbert Perrimon for allowing SG to use facilities at HMS to complete portions of this work. Cliff Tabin and Yohanns Bellaiche gave helpful comments on the manuscript. Thanks to all members of the Mahowald lab for their patience and help. This work was supported by NSF IBN-93006159 to A.P.M. and NIH Predoctoral Fellowship T32 GM07197 to S.G.

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Received for publication March 5, 1996
Accepted May 23, 1996